

**UTILITY APPLICATION**

on

**FLP-MEDIATED GENE MODIFICATION IN MAMMALIAN CELLS, AND  
COMPOSITIONS AND CELLS USEFUL THEREFOR**

by

Geoffrey M. Wahl and Stephen V. O'Gorman

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Attorneys

Foley & Lardner

P.O. Box 80278

San Diego, CA 92138-0278

**FLP-MEDIATED GENE MODIFICATION IN MAMMALIAN CELLS,  
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**RELATED APPLICATIONS**

[0001] This application is a divisional application of U.S. patent application Ser. No. 08/147,912, filed November 3, 1993, currently pending, which is in turn a continuation application of U.S. patent application Ser. No. 07/666,252, filed on March 8, 1991, now abandoned, the entire contents of each of which are hereby incorporated by reference herein. This application is related to U.S. patent application Ser. No. 08/484,324, filed June 7, 1995, now issued as U.S. Patent No. 5,654,182; U.S. patent application Ser. No. 08/486,409, filed June 7, 1995, now issued as U.S. Patent No. 5,677,177; and U.S. patent application Ser. No. 08/825,784, filed April 8, 1997, now issued as U.S. Patent No. 5,885,836; the entire contents of each of which are also incorporated by reference herein.

**FIELD OF THE INVENTION**

[0002] This invention relates to recombinant DNA technology. In a particular aspect, this invention relates to methods for the site-specific recombination of DNA in mammalian cells or host mammalian organisms. In another aspect, the present invention relates to novel DNA constructs, as well as compositions, cells and host organisms containing such constructs. In yet another aspect, the present invention relates to methods for the activation and/or inactivation of expression of functional genes. In a further aspect, the present invention relates to methods for the introduction of DNA into specific sites in the genome of mammalian cells. In a still further aspect, the present invention relates to gene therapy methods. In still another aspect, the present invention relates to means for the recovery of transfected DNA from a cell or host organism. In a still further aspect, the present invention relates to assay methods.

## BACKGROUND OF THE INVENTION

[0003] Many recent manipulations of gene expression involve the introduction of transfected genes (transgenes) to confer some novel property upon, or to alter some intrinsic property of, mammalian cells or organisms. The efficacy of such manipulations is often impaired by such problems as the inability to control the chromosomal site of transgene integration; or the inability to control the number of copies of a transgene that integrate at the desired chromosomal site; or by difficulties in controlling the level, temporal characteristics, or tissue distribution of transgene expression; or by the difficulty of modifying the structure of transgenes once they are integrated into mammalian chromosomes.

[0004] Transgenes are often introduced into mammalian cells or organisms to determine which components of a transgene are required for specific qualitative or quantitative alterations of the host system. Since both chromosomal position and copy number are major determinants of transgene function, the usefulness of these analyses is limited because current techniques for efficiently introducing transgenes into mammalian hosts result in the insertion of a variable number of transgene copies at random chromosomal positions. It is, therefore, difficult (if not impossible) to compare the effects of one transgene to those of another if the two transgenes occupy different chromosomal positions and are present in the genome at different copy numbers. Considerably more refined analyses would be possible if one could routinely introduce single copies of a variety of transgenes into a defined chromosomal position.

[0005] The spatial or temporal characteristics of transgene expression is difficult to control in intact organisms. The restricted expression of transgenes is potentially of great interest, as this technique can be employed for a variety of therapeutic applications, e.g., for the selective interruption of a defective gene, for the alteration of expression of a gene which is otherwise over-expressed or under-expressed, for the selective introduction of a gene whose product is desirable in the host, for the selective removal or disruption of a gene whose expression is no longer desired in the host, and the like.

[0006] Transgene expression is typically governed by a single set of control sequences, including promoters and enhancers which are physically linked to the

transgenes (i.e., cis-acting sequences). Considerably greater expression control could be achieved if transgene expression could be placed under the binary control of these cis-acting sequences, plus an additional set of sequences that were not physically linked to the transgenes (i.e., trans-acting sequences). A further advantage would be realized if the transient activity of these trans-acting functions resulted in a stable alteration in transgene expression. In this manner, it would be possible, for example, to introduce into a host a transgene whose expression would have lethal or deleterious effects if it was constitutively expressed in all cells. This would be accomplished by delaying the expression of the transgene to a specific time or developmental stage of interest, or by restricting the expression of the transgene to a specific subset of the cell population.

[0007] It is currently difficult (if not impossible) to precisely modify the structure of transgenes once they have been introduced into mammalian cells. In many applications of transgene technology, it would be desirable to introduce the transgene in one form, and to then be able to modify the transgene in a defined manner. By this means, transgenes could be activated or inactivated or the sequences which control transgene expression could be altered by either removing sequences present in the original transgene or by inserting additional sequences into the transgene.

[0008] Previous descriptions of recombinase-mediated rearrangement of chromosomal sequences in *Drosophila* and mammalian cells have not directly addressed the question of whether site-specific recombinases could routinely create a functional translational reading frame. Moreover, the reported efficiency of the prior art recombinase system, in the only other description of site-specific recombination in mammalian cells reported to date (based on Cre recombinase, described by Sauer and Henderson, *Nucl. Acids Res.* 17:147, 1989) appears to be quite low.

#### BRIEF DESCRIPTION OF THE INVENTION

[0009] In accordance with the present invention, we have developed a system for the selective modification of chromosomal or extrachromosomal DNA in mammalian cells. Selective modification can involve the insertion of one DNA into another DNA (e.g., to create a hybrid gene, to activate a gene, to inactivate a gene, and the like), or the removal

of specific DNA molecule(s) from other DNA molecule(s) containing the DNA to be removed (e.g., to inactivate a gene, to bring desired DNA fragments into association with one another, and the like).

[0010] The recombination system of the present invention is based on site-specific recombinase, FLP. In one application of the invention recombination system, FLP-mediated removal of intervening sequences is required for the formation of a functional gene. Expression of the functional gene therefore, falls under the control of both the regulatory sequences associated with the functional gene and also under the control of those sequences which direct FLP expression.

[0011] The reverse of the above-described process, i.e., the FLP-mediated introduction of DNA, provides a convenient and selective means to introduce DNA into specific sites in mammalian chromosomes.

[0012] FLP-mediated recombination of marker genes provides a means to follow the fate of various sequences over the course of development and/or from generation-to-generation. The recombination event creates a functional marker gene. This gain-of-function system can be used for lineage analyses in a wide variety of tissues in different organisms. Prior to FLP-mediated recombination, the marker gene is normally silent, i.e., the phenotype typical of the marker is not observed. In the absence of FLP, spontaneous recombination to produce functional marker occurs only at very low frequencies. In the presence of FLP, functional marker is efficiently produced. In addition, this gain-of-function system is heritable and is easily detected by simple histochemical assays. For example, in transgenic mice, the lineages in which recombination is to occur can be controlled by appropriate selection of the promoters used to drive FLP expression. This could include promoters that are only transiently active at a developmental stage that substantially precedes overt cell differentiation. Since transcription of the marker gene is controlled by regulatory sequences associated therewith, functional marker genes can be expressed at later developmental stages, after cell differentiation has occurred. By this means, it is possible to construct a map for mammalian development that correlates embryonic patterns of gene expression with the organization of mature tissues.

## BRIEF DESCRIPTION OF THE FIGURES

[0013] Figure 1 presents schematic diagrams of FLP-mediated recombination events. In Figure 1A, FLP-mediated introduction of DNA is illustrated, while in Figure 1B, FLP-mediated removal of intervening sequences is illustrated.

[0014] Figure 2 is presented in three parts. Figure 2A presents schematic diagrams of the expression vectors pFRT $\beta$ GAL, pNEO $\beta$ GAL, and pOG44 FLP. Figure 2B presents a Southern blot of Hirt lysates prepared from 293 (human embryonic kidney) cells transfected with one microgram of pNEO $\beta$ GAL and varying amounts of the pOG44 FLP expression vector. Figure 2C graphically presents the  $\beta$ -galactosidase activities in the same transfections shown in part B, referred to above.

[0015] Figure 3A, at the top, presents a schematic of the pattern of plasmid integration in E25 deduced from Southern blot analysis. Figure 3A, in the middle, presents the predicted pattern for  $\beta$ -galactosidase positive subclones of E25 if precise recombination across the FLP-recombination target sites occurs. Figure 3A, at the bottom, presents the predicted pattern for  $\beta$ -galactosidase negative, neomycin resistant subclones of E25B2 after FLP mediated insertion of pOG45. Figure 3B presents an analysis of genomic DNA from a cell line with a single integrated copy of pNEO $\beta$ GAL (i.e., CVNEO $\beta$ GAL/E25, designated as E25), two derivative  $\beta$ -galactosidase-positive subclones (designated as E25B1 and E25B2), and two subclones derived from E25B2 after transfection with pOG45 (designated as B2N1 and B2N2).

## DETAILED DESCRIPTION OF THE INVENTION

[0016] In accordance with the present invention, there is provided a mammalian recombination system comprising:

- (i) FLP recombinase, or a nucleotide sequence encoding same, and
- (ii) a first DNA comprising a nucleotide sequence containing at least one FLP recombination target site.

[0017] In accordance with another embodiment of the present invention, there are provided novel DNA constructs useful for the introduction of DNA into the genome of a transfected organism, said DNA construct comprising, as an autonomous fragment:

- (a) at least one FLP recombination target site,
- (b) at least one restriction endonuclease recognition site,
- (c) at least one marker gene,
- (d) a bacterial origin of replication, and optionally
- (e) a mammalian cellular or viral origin of DNA replication.

[0018] In accordance with yet another embodiment of the present invention, there are provided novel DNA constructs useful for the rescue of DNA from the genome of a transfected organism, said DNA construct comprising, as an autonomous fragment, in the following order, reading from 5' to 3' along said fragment:

- (a) a first FLP recombination target site,
- (b) an insert portion comprising, in any suitable sequence:
  - (1) at least one restriction endonuclease recognition site,
  - (2) at least one marker gene,
  - (3) a bacterial origin of replication, and optionally
  - (4) a mammalian cellular or viral origin of DNA replication, and
- (c) a second FLP recombination target site in tandem with said first FLP recombination target site.

In addition, there are provided methods for the recovery of transfected DNA from the genome of a transfected organism employing the above-described constructs.

[0019] In accordance with still another embodiment of the present invention, there is provided a method for the assembly of a functional gene (which is then suitable for activation of expression), in mammalian cells, by recombination of individually inactive gene segments derived from one or more gene(s) of interest, wherein each of said segments contains at least one recombination target site, said method comprising: contacting said individually inactive gene segments with a FLP recombinase, under conditions suitable for recombination to occur, thereby providing a DNA sequence which encodes a functional gene of interest.

[0020] In accordance with a further embodiment of the present invention, there is provided a method for the disruption of functional gene(s) of interest, thereby inactivating expression of such gene(s), in mammalian cells, wherein said gene(s) of interest contain at least one FLP recombination target site, said method comprising contacting said gene(s) of interest with:

- (i) a DNA segment which contains at least one FLP recombination target site,  
and
- (ii) FLP recombinase;

wherein said contacting is carried out under conditions suitable for recombination to occur between said gene and said DNA segment, thereby disrupting the gene(s) of interest and rendering said gene(s) non-functional.

[0021] In accordance with a still further embodiment of the present invention, there is provided a method for the precisely targeted integration of DNA into the genome of a host organism, said method comprising:

- (i) introducing a FLP recombination target site into the genome of cells which are compatible with the cells of the subject,
- (ii) introducing a first DNA comprising a nucleotide sequence containing at least one FLP recombination target site therein into the FLP recombination target site in the genome of said cells by contacting said cells with said first DNA and FLP recombinase, and thereafter
- (iii) introducing the cells produced by the process of step (ii) into said subject, have the optional ability to have DNA reproducibly and repetitively inserted into and/or recovered from the host cells and/or organism.

[0022] In accordance with another aspect of the present invention, there are provided mammalian cells, wherein the genomic DNA of said cells contain at least one FLP recombination target site therein.

[0023] In accordance with yet another aspect of the present invention, there are provided transgenic, non-human mammals, wherein said mammals contain at least one FLP recombination target site in the genomic DNA thereof.

[0024] In accordance with yet another aspect of the present invention, there is provided a method for the site-specific integration of transfected DNA into the genome of the above-described cells and/or transgenic, non-human mammals, said method comprising:

- (i) contacting said genome with:
  - (a) FLP recombinase, and
  - (b) a first DNA comprising a nucleotide sequence containing at least one FLP recombination target site therein; and thereafter
- (ii) maintaining the product of step (i) under conditions suitable for site-specific integration of said DNA sequence to occur at the FLP recombination target site in said genome.

[0025] In accordance with a further aspect of the present invention, there is provided a method for the analysis of the development of a mammal, said method comprising:

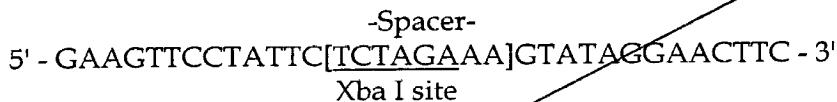
- (a) providing a transgenic mammal comprising:
  - (i) an expression construct encoding FLP under the control of a conditional promoter, and
  - (ii) a reporter construct under the control of the same or a different promoter, wherein said reporter construct encodes a functional or non-functional reporter gene product, and wherein said construct contains at least one FLP recombination target site therein,
    - wherein the functional expression of the functional reporter gene is disrupted when said FLP recombination event occurs, or
    - wherein the functional expression of the non-functional reporter gene commences when said FLP recombination event occurs; and
- (b) following the development of said mammal to determine when expression of functional reporter gene product either commences or is disrupted.

[0026] In accordance with a still further aspect of the present invention, there is provided a co-transfection assay FLP-mediated recombination, said assay comprising:

- (a) co-transfected a host mammalian cell with:
  - (i) a FLP expression plasmid, and
  - (ii) a reporter plasmid comprising a reporter gene inactivated by the presence of at least one recombination target site; and
- (b) monitoring said host cell under a variety of conditions for the gain of expression of functional reporter gene product.

[0027] FLP recombinase is a protein which catalyzes a site-specific recombination reaction that is involved in amplifying the copy number of the 2 $\mu$  plasmid of *S. cerevisiae* during DNA replication. FLP protein has been cloned and expressed in *E. coli* (see, for example, Cox, *Proc. Natl. Acad. Sci. U.S.A.*, 80:4223-4227, 1983), and has been purified to near homogeneity (see, for example, Meyer-Lean et al., *Nucl. Acids Res.*, 15:6469-6488, 1987). FLP recombinases contemplated for use in the practice of the present invention are derived from species of the genus *Saccharomyces*. Preferred recombinases employed in the practice of the present invention are derived from strains of *Saccharomyces cerevisiae*. Especially preferred recombinases employed in the practice of the present invention are proteins having substantially the same amino acid sequence as set forth in SEQ ID NO: 2, as encoded, for example, by SEQ ID NO: 1, or the sequence set forth by Hartley and Donelson, *Nature* 286: 860, 1980.

*Subseq a'* [0028] The FLP recombination target site (sometimes referred to herein as "FRT") has also been identified as minimally comprising two 13 base-pair repeats, separated by an 8 base-pair spacer, as follows:



The nucleotides in the above "spacer" region can be replaced with any other combination of nucleotides, so long as the two 13 base-pair repeats are separated by 8 nucleotides. The actual nucleotide sequence of the spacer is not critical, although those of skill in the art recognize that, for some applications, it is desirable for the spacer to be asymmetric, while for other applications, a symmetrical spacer can be employed. Generally, the spacers of the FLP recombination target sites undergoing recombination with one another will be the same.

[0029] As schematically illustrated in Figure 1A, contact of genomic DNA containing a FLP recombination target site (shown as the linear Psv-BETA-GAL construct) with a vector containing a FLP recombination target site, in the presence of the protein, FLP recombinase, results in recombination that forms a new genomic sequence wherein the vector sequences have been precisely incorporated into the genome of the host. The reverse of this process is shown schematically in Figure 1B, wherein a genomic sequence or construct containing two tandemly oriented FLP recombination target sites, upon contacting with FLP, is recombined and the FLP recombination target site-bounded fragment is excised as a circular molecule.

[0030] Genes of interest contemplated for use in the practice of the present invention can be selected from genes which provide a readily analyzable functional feature to the host cell and/or organism, e.g., visible markers (such as  $\beta$ -galactosidase, thymidine kinase, tyrosinase, and the like), selectable markers, (such as markers useful for positive and negative selection, e.g., genes for antibiotic resistance), as well as other functions which alter the phenotype of the recipient cells, and the like.

[0031] The first DNA employed in the practice of the present invention can comprise any nucleotide sequence containing at least one FLP recombination target site, which will precisely define the locus at which FLP-mediated recombination will occur. The nucleotide sequence can comprise all or part of a gene of interest, as well as other sequences not necessarily associated with any known gene. Optionally, for ease of later recovery of the gene of interest (in "activated" or modified form), the first DNA can optionally contain a second FLP recombination target site.

[0032] The second DNA employed in the practice of the present invention is selected from at least a second portion of the first gene of interest or at least a portion of a second gene of interest (including an intact form of a second gene of interest). When the second DNA is at least a second portion of the first gene of interest, the site-specific recombination of the present invention may act to provide a functional combination of the different portions of the first gene of interest. Alternatively, when the second DNA is at least a portion of a second gene of interest, the site-specific recombination of the present invention may act to provide a functional hybrid gene, which produces a product which is not identical with either the product of the first gene or the second

gene. As yet another alternative, when the second DNA is a portion of a second gene, the site-specific recombination of the present invention may act to disrupt the function of the first gene of interest. Based on the nature of the first DNA and the second DNA, as well as the mode of interaction between the two, the site-specific interaction of the present invention may create or disrupt a feature which is colorimetrically detectable, immunologically detectable, genetically detectable, and the like.

[0033] In accordance with the present invention, assembly of a functional expression unit is achieved in any of a variety of ways, e.g., by association of the gene of interest with a functional promoter, by assembly of common gene fragments to produce a complete functional gene (which, in combination with its promoter, comprises a functional expression unit), or assembly of diverse gene fragments from diverse sources to produce a functional, hybrid gene (which, in combination with a promoter, comprises a functional expression unit), and the like. Upon assembly of a functional expression unit as described herein, expression of the functional gene to produce a protein product can be activated in the usual manner. In the absence of FLP-mediated recombination, activation of expression would fail to produce a functional protein product.

[0034] In accordance with the present invention, dis-assembly of a functional expression unit is achieved in any of a variety of ways, e.g., by dis-associating the gene of interest from a functional promoter, by disassembly (e.g., disruption) of the functional gene (e.g., by introduction of DNA which renders the entire sequence non-functional), by removal of a substantial portion of the coding region of said gene, and the like. Upon dis-assembly of a functional expression unit as described herein, expression of the functional gene product under the conditions required prior to gene dis-assembly is no longer possible. The ability of the expression unit to be activated for expression has therefore been disrupted. The gene in this situation can be said to be inactivated, since activation of expression is not possible.

[0035] Individually inactive gene segments contemplated for use in the practice of the present invention are fragments which, alone, do not encode functional products. Such fragments can be derived from a first gene of interest alone, or from both a first and second gene of interest DNA fragments.

[0036] When gene inactivation is desired, the gene of interest can be disrupted with a DNA fragment which throws the gene of interest out of reading frame (e.g., an insert wherein the number of nucleotides is not a multiple of 3). Alternatively, the gene of interest can be disrupted with a fragment which encodes a segment which is substantially dissimilar with the gene of interest so as to render the resulting product non-functional. As yet another alternative, the gene of interest can be disrupted so as to dis-associate the gene of interest from the transcriptional control of the promoter with which it is normally associated.

[0037] The introduction of DNA, e.g., DNA encoding FLP recombination target sites, into the genome of target cells can be accomplished employing standard techniques, e.g., transfection, microinjection, electroporation, infection with retroviral vectors, and the like.

[0038] Introduction of protein, e.g., FLP recombinase protein, to host cells and/or organisms can be accomplished by standard techniques, such as for example, injection or microinjection, transfection with nucleotide sequences encoding FLP, and the like.

[0039] When employed for gene therapy of an intact organism, introduction of transgenic cells into the subject is accomplished by standard techniques, such as for example, grafting, implantation, and the like.

[0040] Mammalian cells contemplated for use in the practice of the present invention include all members of the order Mammalia, such as, for example, human cells, mouse cells, rat cells, monkey cells, hamster cells, and the like.

[0041] Host organisms contemplated for use in the practice of the present invention include each of the organism types mentioned above, with the proviso, however, that no claim is made to genetically modified human hosts (although the present invention contemplates methods for the treatment of humans).

[0042] Once FLP recombinase (or DNA encoding same) and DNA containing at least one FLP recombination target site have been introduced into suitable host cells/organisms, the cells/host organisms are maintained under conditions suitable for the site-specific recombination of DNA. Such conditions generally involve conditions

required for the viability of the host cell or organism. For in vitro manipulations, conditions employed typically involve low concentrations of a variety of buffers having a pH of between about 5-9 and ionic strengths in the range of about 50-350 mM. See, for example, Senecoff et al., *J. Mol. Biol.*, 201:405-421, 1988.

[0043] In accordance with a particular aspect of the present invention, a co-transfection assay has been developed which can be used to characterize FLP-mediated recombination of extrachromosomal DNA in a variety of cell lines. Cells are co-transfected with an expression construct and a "reporter" plasmid that is a substrate for the recombinase. The expression construct encodes a FLP recombinase protein. The reporter plasmid encodes either a functional reporter gene containing at least one recombination target site therein, or a non-functional reporter gene containing at least one recombination target site therein. Upon expression of FLP by the expression construct, the functional reporter gene will be rendered non-functional, or the non-functional reporter gene will be rendered functional. Thus, the activity of the expression construct can be assayed either by recovering the reporter plasmid and looking for evidence of recombination at the DNA level, or by preparing cytoplasmic extracts and looking for evidence of recombination at the protein level (i.e., by measuring the expression of reporter gene activity generated by the recombined reporter). Such assays are described in greater detail in Example 1 below.

[0044] The invention will now be described in greater detail by reference to the following non-limiting examples.

## EXAMPLES

### Example 1. Co-transfection Assays.

*better  
DNA's  
are  
from* [0045] The co-transfection assay used to characterize FLP-mediated recombination of extrachromosomal DNA involved transfection of cells with an expression construct and a "reporter" plasmid that was a substrate for the recombinase. The activity of the expression construct could be assayed either by recovering the reporter plasmid and looking for molecular evidence of recombination at the DNA level, or by preparing

cytoplasmic extracts and looking for evidence of recombination at the protein level (i.e., by measuring  $\beta$ -galactosidase activity generated by recombined reporter).

[0046] The pNEO $\beta$ GAL reporter plasmid used for these assays was derived from pFRT $\beta$ GAL (Fig. 2A). In the Figure, half-arrows indicate positions of FLP recombination target (FRT) sites; E and S designate EcoRI and SacI restriction sites, respectively; Psv designates early promoter from SV40; BETA-GAL designates the  $\beta$ -galactosidase structural sequence; NEO designates neomycin expression cassette; Pcmv designates the cytomegalovirus immediate early promoter; IN designates an intron; FLP designates a FLP coding sequence; AN designates an SV40 adenylation cassette; thin lines represent vector sequences; and the sizes of restriction fragments are indicated in kb.

*sub 2*  
 [0047] pFRT $\beta$ GAL contains a version of the bacterial  $\beta$ -galactosidase sequence modified by insertion of a FLP recombination target site, or FRT, within the protein coding sequence immediately 3' to the translational start. The oligonucleotide used for the construction of pFRT $\beta$ GAL was:

5' - GATCCGGGCTACCATGGA GAAGTTCTATT CGAAGTTCTATT  
(TCTAGA)AAGTATAAGAACCTCA - 3'.

This oligonucleotide contains an in-frame start codon, minimal FRT site, and an additional copy of the 13-bp FRT repeat [°XXX°]; the XbaI site within the FRT spacer is enclosed in parentheses. The linker was inserted between the BamHI and HindIII sites of pSKS105 (Casadaban et al., *Meth. Enzymol.* 100:293, 1983) and the LacZ portion of modified gene was cloned into a pSV2 vector. The neomycin cassette used for construction of pNEO $\beta$ GAL was an XhoI to BamHI fragment from pMC1neo-polyA (Thomas and Capecchi, *Cell* 51:503, 1987) cloned between copies of the J3 FRT site in pUC19.

[0048] The FRT consists of two inverted 13-base-pair (bp) repeats and an 8-bp spacer that together comprise the minimal FRT site, plus an additional 13-bp repeat which may augment reactivity of the minimal substrate. The  $\beta$ -galactosidase translational reading frame was preserved upon insertion of the FRT site, and the resulting plasmid, pFRT $\beta$ GAL, generated robust activity in mammalian cells (Table 1).

[0049] pNEO $\beta$ GAL was constructed by cutting pFRT $\beta$ GAL in the middle of the FRT site with XbaI and then inserting an XbaI fragment consisting of two half-FRT sites flanking a neomycin transcription unit. This created intact FRTs on either side of the neomycin cassette and rendered the  $\beta$ -galactosidase transcription unit inactive (Table 1). Precise FLP-mediated recombination of the FRTs caused the excision of the neomycin cassette, recreated the parental pFRT $\beta$ GAL plasmid, and restored  $\beta$ -galactosidase expression.

[0050] Co-transfection of cells with a fixed amount of pNEO $\beta$ GAL reporter plasmid and increasing amounts of the pOG44 FLP expression vector generated increasing amounts of recombined reporter plasmid and consequently, increased levels of  $\beta$ -galactosidase activity. Molecular evidence for FLP-mediated recombination was obtained by recovering plasmids 36 hours after transfection, followed by endonuclease treatment (with EcoRI and Scal) and Southern blotting (see Fig. 2B; employing as a probe the fragment of pFRT $\beta$ GAL indicated at the top of Fig. 2A). Lysates of cells from cotransfections that included the pOG44 FLP expression vector showed a signal at 5.6 kb, the position at which recombined reporter (equivalent to pFRT $\beta$ GAL) would run, and a 3.2 kb signal that was generated by unrecombined pNEO $\beta$ GAL reporter (Fig. 2A). The 5.6 kb band intensity was proportional to the amount of FLP expression plasmid included in the transfection. The 5.6 kb band was not seen in cotransfections in which a non-FLP plasmid was substituted for the FLP expression vector (Fig. 2B) or in transfections that contained only pOG44 (and no reporter plasmid). pOG44 generated additional signals at 2.2 kb and 2.8 kb because the plasmid used in its construction contained EcoRI and EcoRI-Scal fragments of such length.

[0051] pOG44 consists of the cytomegalovirus immediate early promoter from pCDM8 (see Aruffo and Seed, *Proc. Natl. Acad. Sci. USA* 84:8573, 1987), a 5' leader sequence and synthetic intron from pMLSIScat (see Huang and Gorman, *Nucl. Acids Res.* 18: 937, 1990), the FLP coding sequence (bp 5568-6318 and 1-626 of the 2 $\mu$ m circle, (see Hartley and Donelson, *Nature* 286:860, 1980) and the SV40 late region polyadenylation signal from pMLSIScat. The following silent nucleotide substitutions were introduced into the structural FLP sequence using the polymerase chain reaction: C for T at position 5791, G for A at 5794, G for C at 5800, C for T at 55, G for A at 58, and C for T at 103.

These changes eliminated three canonical AATAAA polyadenylation signals and introduced a PstI restriction site without altering the amino acid sequence encoded by the nucleotide sequence. pOG28 consists of a murine cDNA for dihydrofolate reductase cloned into pCDM8 (Aruffo and Seed, *supra*).

[0052] In the same samples,  $\beta$ -galactosidase activity was also proportional to the amount of FLP expression plasmid included (Fig. 2C). Only background activities were observed in cotransfections that included a non-FLP control plasmid (Table 1) or when pOG44 alone was transfected. The experiment thus provides both molecular and biochemical evidence for precise FLP-mediated recombination in mammalian cells.

[0053] Table 1 presents  $\beta$ -galactosidase activities in cotransfection assays of 293, CV-1, and F9 cells. Positive control transfections (pFRT $\beta$ GAL) included 1  $\mu$ g of pFRT $\beta$ GAL and 18  $\mu$ g of the pOG28 non-FLP control plasmid; negative control transfections (pNEO $\beta$ GAL) included 1  $\mu$ g of pNEO $\beta$ GAL and 18  $\mu$ g of the pOG28; and experimental transfections (pNEO $\beta$ GAL + FLP) contained 1  $\mu$ g of pNEO $\beta$ GAL and 18  $\mu$ g of the pOG44 FLP expression plasmid (Fig. 1A). The column headed by "%" shows the pNEO $\beta$ GAL + FLP values as a percentage of the pFRT $\beta$ GAL positive control. Each value represents the mean for six plates from two experiments. Standard errors are in parentheses. Neither pOG28 nor pOG44 generated  $\beta$ -galactosidase activity when transfected alone. All transfections contained 1  $\mu$ g of pRSVL (de Wet et al., *Mol. Cell. Biol.* 7:725, 1987) to correct  $\beta$ -galactosidase activities for relative transfection efficiencies.

[0054] Subconfluent cultures of cells in 10 cm dishes and grown in Dulbecco's modified Eagle's medium (DMEM) and 5% calf serum were transfected by overnight exposure to calcium phosphate precipitates (Graham et al., *Virology* 36:59, 1979) and then split 1:4. After 24 hours incubation, one plate of each transfection was harvested by Hirt extraction (*J. Mol. Biol.* 26:365, 1967) and a second plate was used to prepare cytoplasmic extracts (de Wet et al., *supra*). Approximately 5% of the DNA recovered from single plates was used for Southern analyses.  $\beta$ -galactosidase assays were performed as described by Hall et al., *J. Mol. Appl. Genet.* 2:101, 1983). Luciferase activities generated by the inclusion of 1  $\mu$ g of pRSVL (de Wet et al., *supra*) in all transfections were used to correct  $\beta$ -galactosidase activities for relative transfection efficiencies. The experiment was repeated twice with similar results.

TABLE 1:  $\beta$ -GALACTOSIDASE ACTIVITIES (UNITS/ MG PROTEIN) IN COTRANSFECTED CELLS

<u>CELL LINE</u>	<u>TRANSFECTIONS</u>			<u>%</u>
	<u>pFRT<math>\beta</math>GAL</u>	<u>pNEO<math>\beta</math>GAL</u>	<u>pNEO<math>\beta</math>GAL + FLP</u>	
293	30.4 (1.9)	0.17 (0.02)	14.2 (2.2)	47
CV-1	275 (25)	0.33 (0.06)	22.6 (1.2)	8
F9	24.8 (4.3)	0.04 (0.01)	1.88 (0.02)	8

[0055] FLP activity has also been demonstrated in monkey kidney (CV-1) and mouse embryonal carcinoma (F9) cells. In Table 1, the  $\beta$ -galactosidase activity in the "pFRT $\beta$ GAL" transfections represents an estimate of the expression expected if all the pNEO $\beta$ GAL in a co-transfection were immediately recombined. The highest  $\beta$ -galactosidase expression in co-transfections employing pNEO $\beta$ GAL plus pOG44, relative to pFRT $\beta$ GAL transfected cells, was 47%, seen in 293 cells. This is a remarkable level considering that  $\beta$ -galactosidase expression required both FLP expression, followed by recombination of pNEO $\beta$ GAL, to produce a construct capable of expressing  $\beta$ -galactosidase. Co-transfections of CV-1 and F9 cells generated 8% of the activity seen in the pFRT $\beta$ GAL transfections. Even at this lower relative activity, cotransfected cells were readily observed in histochemical reactions for  $\beta$ -galactosidase activity.

#### Example 2. FLP-Mediated Removal of Intervening Sequences.

[0056] If the invention method is to be widely applicable, for example for gene activation in transgenic mammals, the ability of FLP to faithfully promote precise recombination at FLP recombination target sites contained in the mammalian genome is required. Such ability is demonstrated in this example.

[0057] Cell lines that contain single integrated copies of pNEO $\beta$ GAL (designated CVNEO $\beta$ GAL/E) were produced by transfecting CV-1 cells with linearized plasmid by electroporation, then isolated by selecting G418-resistant (G418<sup>R</sup>) transfecants that stably expressed the neomycin cassette, and finally identifying single copy lines by Southern blot analyses (Fig. 3). As previously shown for other integrated constructs with similarly short direct repeats, the chromosomal FRTs did not spontaneously

recombine (in the absence of FLP) to produce a  $\beta$ -galactosidase-positive ( $\beta$ GAL $^+$ ) phenotype at detectable frequencies (Table 2).

[0058] Transient expression of FLP in the CVNEO $\beta$ GAL/E lines (by transiently transfecting with the pOG44 FLP expression vector) promoted a rapid conversion to a  $\beta$ GAL $^+$  phenotype. When five different lines were transiently transfected with the pOG44 FLP expression vector,  $\beta$ -galactosidase activities at 36 hours were 40 to 100-fold higher than those seen in replicate plates transfected with a non-FLP plasmid (Table 2). At 48 hours after transfection histochemical processing showed many positive cells (Table 2).

[0059] Table 2 presents the  $\beta$ -galactosidase phenotypes of CVNEO $\beta$ GAL/E lines, which contain a single copy of the  $\beta$ -galactosidase inactive reporter, pNEO $\beta$ GAL, after transfection with FLP expression (pOG44), non-FLP negative control (pOG28) or  $\beta$ -galactosidase positive control (pFRT $\beta$ GAL) plasmids. The pFRT $\beta$ GAL transfections included 1  $\mu$ g of pFRT $\beta$ GAL and 19  $\mu$ g of pOG44; other mixes contained 20  $\mu$ g of the indicated plasmid.  $\beta$ -galactosidase activities are mean values for triplicate transfections performed as described for Fig. 2 and assayed 36 hours after removal of precipitates; standard errors for the pOG44 transfections were less than 10% of the mean. The percent positive was determined by scoring more than  $10^3$  cells after transfection and histochemical processing as described by de Wet et al., *supra*.

TABLE 2:  $\beta$ -galactosidase PHENOTYPES OF TRANSFECTED CVNEO $\beta$ GAL CELL LINES

CELL LINE	ACTIVITIES (units/mg protein)		PERCENT POSITIVE		
	pOG28	pOG44	pOG28	pFRT $\beta$ GAL	pOG44
E6	0.24	11.2	0*	8.7	6.1
E25	0.21	16.7	0*	17.1	12.4
E26	0.18	7.2	0*	19.5	15.4
E14	0.28	13.1	ND	ND	ND
E22	0.09	9.6	ND	ND	ND

\* No positive cells were found among  $>10^6$  cells examined.

ND: Not done.

[0060] To provide some estimate of the efficiency of recombination, an additional set of replicate plates were transfected with the pFRT $\beta$ GAL  $\beta$ -galactosidase expression vector. Comparing the fractions of cells that were  $\beta$ GAL $^+$  in the pFRT $\beta$ GAL and in the pOG44 transfections (assuming similar transfection efficiencies) suggests that most (70-80%) of the cells transfected with pOG44 were converted to a  $\beta$ GAL $^+$  phenotype (Table 2). The comparison undoubtedly underestimates the efficiency of FLP-mediated excision. Whereas many copies of a functional  $\beta$ -galactosidase gene were available for immediate transcription in the positive controls, recombination may have occurred shortly before harvest in some pOG44-transfected cells. In these cases the single recombined reporter gene may not have generated enough  $\beta$ -galactosidase by the time of harvest to render the cells positive in this assay.

[0061] The  $\beta$ GAL $^+$  phenotype was passed on to all descendants of many FLP-converted cells. Positive colonies were formed during prolonged expansion of individual colonies. Entirely negative colonies and mixed colonies were also observed. Mixed colonies would be expected if recombination occurred after mitosis in only one descendent of a transfected cell, or if recombinant and unrecombinant cells mixed at replating or during subsequent growth. Indeed, the physical segregation of phenotypes evident in most mixed colonies suggested that they were composed of stably positive and negative lineages.

[0062] The correlation between  $\beta$ -galactosidase expression and recombination at FRT sites was examined by comparing the structure of the integrated pNEO $\beta$ GAL sequences in two  $\beta$ GAL $^+$  subclones to the parental line. CVNEO $\beta$ GAL/E25 (106) cells were transfected with the pOG44 FLP expression vector and subcloned 12 hours after removal of the precipitate. After histochemical screening, two  $\beta$ GAL $^+$  subclones (E25B1 and E25B2) were expanded for further analysis. In Southern blots of genomic DNA from both subclones, the pattern of hybridization matched that expected for FLP-mediated recombination of the FRT sites in the parental line (Fig. 3). While recombination products have not been recovered and sequenced, these Southern analyses and the fact that activation of  $\beta$ -galactosidase expression required creation of a functional translational reading frame indicate that FLP-mediated recombination was precise.

Example 3. FLP Mediated Recombination of FRT on an Extrachromosomal Molecule  
With a Chromosomally Integrated FRT.

[0063] Reversal of the process described in the previous Example, i.e., the FLP-mediated recombination of an FRT site on a plasmid with a chromosomally integrated FRT site, can be used to target the integration of transfected plasmids to specific genomic sites. To determine the frequency at which this occurs, G418-sensitive,  $\beta$ GAL<sup>+</sup> E25B2 cells were co-transfected with the pOG44 FLP expression vector and a plasmid, pOG45, that contained a neomycin resistance gene expression cassette and a single FRT. pOG45 consisted of the neomycin resistance cassette and 3' FRT from pNEO $\beta$ GAL cloned into pUC19.  $8 \times 10^5$  CVNEO $\beta$ GAL cells were transfected by electroporation in 800  $\mu$ l of saline containing 40  $\mu$ g of pOG44 and 0.1  $\mu$ g of either pOG45 or, for a negative control, pOG45A (which was derived from pOG45 by deleting a 200 bp fragment containing the FRT).

[0064] G418<sup>R</sup> subclones (designated B2N) from three transfections that had stably integrated pOG45 were histochemically stained for  $\beta$ -galactosidase activity and more than half (104 of 158, or ~66%) were either entirely  $\beta$ -galactosidase-negative ( $\beta$ GAL<sup>-</sup>) or predominantly  $\beta$ GAL<sup>-</sup> with a few clusters of  $\beta$ GAL<sup>+</sup> cells. The remaining colonies were  $\beta$ GAL<sup>+</sup>. With continued passage as dispersed monolayers, the fraction of  $\beta$ GAL<sup>+</sup> cells in the mosaic lines rapidly diminished. This suggested they were G418 sensitive cells that initially survived because of their proximity to resistant cells; this was confirmed by reconstitution experiments. All of the 55 colonies formed after parallel co-transfections of pOG44 and a derivative of pOG45 (pOG45A) that lacked an FRT were  $\beta$ GAL<sup>+</sup>.

[0065] The correlation between loss of  $\beta$ -galactosidase activity and recombination between plasmid and chromosomal FRTs was examined in Southern analyses. Because the FRT and neomycin cassette of pOG45 were derived from the neomycin cassette and 3' FRT of pNEO $\beta$ GAL (Fig. 2A), recombination of the plasmid FRT with the E25B2 chromosomal FRT regenerates the 3.2 kb EcoRI fragment of the original CVNEO $\beta$ GAL/E25 parent. Additionally, the 8.5 kb junctional fragment of CVNEO $\beta$ GAL/E25 shifts to 12.0 kb because pOG45 is 3.5 kb larger than the neomycin

cassette of pNEO $\beta$ GAL. The 3.2 kb EcoRI fragment and the 8.5 kb junctional fragment were observed in each of the 10 cell lines analyzed after initial histochemical classification as  $\beta$ GAL $^-$  or mosaic, as shown for two such lines in Fig. 3B. In contrast, each of the four  $\beta$ GAL $^+$  colonies examined by Southern analyses showed that pOG45 had integrated at a random site.

[0066] These data show that FLP-mediated recombination will target the integration of transfected DNA to a specific chromosomal site at frequencies that exceed those of random integration, and that the event can be marked by the alteration in gene activity at the target site. The efficiency of targeted integration can be increased by standard optimization techniques, such as for example, by using ratios of the integrating plasmid and FLP expression vectors different from the single ratio mixture used here, or by using FRT mutations in the plasmid and chromosomal sites to decrease the frequency with which successfully integrated plasmids are subsequently excised.

[0067] While the invention has been described in detail with reference to certain preferred embodiments thereof, it will be understood that modifications and variations are within the spirit and scope of that which is described and claimed.